

Indels' based genetic sex-differentiation in whiteleg shrimp *Penaeus vannamei*

Diferenciación genética del sexo en el camarón blanco *Penaeus vannamei*
basada en indels

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ABSTRACT

Sex identification in the whiteleg shrimp *Penaeus vannamei* is relevant for improving its aquaculture, either for broodstock management or for monosex line production. In this work, we designed a technique for sex differentiation based on genetic markers. Utilizing long-read sequence databases, we report the identification of indels unique to one sex. Primers designed to differentially amplify DNA sequences for females and males lead to the characterization of the putative haplotypes W and Z, respectively. Indels mapped to a sex-associated region in a specific chromosome. The sex-identification technique, based on simple gel electrophoresis of PCR products, was validated using both cultivated and wild samples, as well as larvae (nauplii and mysis stages). Several sex-related genes were found upstream and downstream of the indels along the same chromosome. Further work is needed to identify potential sex-determining mechanisms on this chromosome.

Keywords: Sex identification, shrimp aquaculture, sex-associated genes, shrimp larvae, insertions/deletions.

RESUMEN

La identificación del sexo en el camarón blanco *Penaeus vannamei* es relevante para el mejoramiento del cultivo, ya sea a través del manejo de los lotes de reproductores o para la producción de líneas monosexo. En este trabajo, diseñamos una técnica para la diferenciación sexual basada en marcadores genéticos. Mediante el uso de bases de datos de secuencias largas, reportamos la identificación de indels únicos a uno de los sexos. El diseño de iniciadores para la amplificación diferencial del ADN de hembras y machos condujo a la caracterización respectiva de los haplotipos putativos W y Z. Los indels mapearon a una región asociada con el sexo de un cromosoma específico. La técnica de identificación del sexo, basada en una sencilla electroforesis en gel de los productos de PCR, se validó utilizando tanto muestras de cultivo como silvestres, así como de larvas (estadios de nauplios y mysis). Se encontraron varios genes relacionados con el sexo río arriba y río abajo a lo largo del cromosoma. Se requiere trabajo futuro para descubrir mecanismos potenciales de determinación del sexo en este cromosoma.

Palabras clave: Identificación de sexo, camaronicultura, genes asociados al sexo, larvas de camarón, inserciones/deleciones.

INTRODUCTION

The whiteleg shrimp *Penaeus (Litopenaeus) vannamei* is a worldwide cultivated species with a production of over 5.8 million tons (FAO, 2022). Cultivation of monosex lines could contribute to increasing farm productivity, as females generally grow faster than males (Chow and Sandifer, 1991; Bardeira *et al.*, 2020; Moss *et al.*, 2002). For this purpose, as well as for broodstock management, studies on sex determination and methods for early sex differentiation are necessary; it is estimated that in *P. vannamei* the sex can be visually differentiated after an age of 90 days (pL60) (Garza-Torres *et al.*, 2009).

Even though there are no evident differences between males and females at the chromosomal level (Campos-Ramos, 1997), several studies have shown that the sex-determination system is ZZ/ZW in which males are homogametic (ZZ), and females heterogametic (WZ) (Alcivar-Warren *et al.*, 2007; Zhang *et al.*, 2007; Yu *et al.*, 2017; Perez-Enriquez *et al.*, 2018; 2020; Jones *et al.*, 2020; Garcia *et al.*, 2024).

Several strategies, relying on the detection of one or more Single Nucleotide Polymorphisms (SNPs) located in putatively DNA sex-linked regions, have been designed for sex identification in *P. vannamei* (Yu *et al.*, 2017; Jones *et al.*, 2020; Wang *et al.*, 2020; Perez-Enriquez *et al.*, 2020). These techniques can be applied to early development stages; thus, sexing can be done before secondary structures appear. However, the reported techniques rely on either DNA sequencing or High-Resolution Melting, which are often not feasible in a basic molecular biology laboratory.

To overcome this, an alternative method would only require an endpoint PCR for DNA amplification and the observation of differences in fragment sizes between sexes using agarose gel electrophoresis. These differences would derive from the detection of indels, rather than SNPs. Such a technique has been reported for *Penaeus monodon* (Staelens *et al.*, 2008), but the reported primers failed to amplify in *P. vannamei* (unpublished data).

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In a study reporting a high-quality *P. vannamei* genome assembly (Perez-Enriquez *et al.*, 2024), our group mapped the sex-markers described in Perez-Enriquez *et al.* (2020) to chromosome 12 (Sequence CM075139.1 in the GenBank assembly GCA_037577135.2). In this study, we report the presence of sex-related indels located near the putatively sex-associated SNPs and designed effective primers to amplify the regions of interest. Furthermore, we document the identification of potential sex-related genes on this chromosome.

MATERIALS AND METHODS

Detection of sex-differentiated indels by *in silico* analysis on DNA sequence databases

An SRA-blast (<https://www.ncbi.nlm.nih.gov/sra>) was done between the reverse-complement of the 577 bp fragment reported in Perez-Enriquez *et al.* (2020) and the Sequel II PacBio long-reads sequences archives (SRR28525434–SRR28525438). Reads with > 99 % similarity were selected and further separated into haplotypes depending on the SNPs present in positions 413, 447, and 448 (Perez-Enriquez *et al.*, 2020). Reads were grouped as “Haplotype W” in which the SNPs were A, A, and T, respectively, for the Plus/Plus alignments and A, T, and T, for the Plus/Minus alignments. Conversely, reads were grouped as “Haplotype Z” in which the SNPs were G, G, and G, respectively, for the Plus/Plus alignments and C, C, and C, for the Plus/Minus alignments. In both cases, the reads were downloaded from NCBI as concatenated FASTA files.

We aligned the FASTA files for each haplogroup to chromosome 12 (CHR_12) of the *P. vannamei* assembly (GCA_037577135.2) with minimap2 ver. 2.17 (Li, 2018) and visualized in JBrowse2 (Diesh *et al.*, 2023) to confirm that each read was completely aligned (> 90 % similitude) to the CHR_12. The reads of each haplogroup were assembled with wtdbg2 (Ruan and Li, 2020) to obtain a consensus sequence. Each consensus sequence was used for genome walking to search for reads that would allow for the assembly of larger fragments for each haplogroup, both upstream and downstream. For this genome walking process, SRA-blast of the consensus sequence and the Sequel II PacBio long-reads sequences archives (SRR28525434–SRR28525438) were carried out. The matching reads were downloaded, aligned to the genome, and visualized in JBrowse2 as described above. The genome walking process was repeated until no further reads could be isolated in the SRA-Blast.

The complete reads for each haplogroup were again aligned to chromosome 12 with minimap2 version 2.17 (Li, 2018), visualized in JBrowse2 (Diesh *et al.*, 2023), and edited for putatively sequencing errors in ChromasPro (Technelysium, Australia). Indels, sequences present in haplotype W and absent in haplotype Z, were visually identified in JBrowse2.

Design and validation of primers to amplify fragments including indels.

Primers were designed in the flanking regions of the identified indels with Primer3 (Koressaar *et al.*, 2018). The expectation was to have two bands for females and one band for males.

DNA samples from twelve females and seven males of wild origin (Mexican Pacific) were used for the amplification. PCR reactions were done in 15 µL volumes with 1.5 µL of 5× Colorless Flexi Buffer (Promega), 1.2 µL of 25 mM MgCl₂ (Promega), 0.2 µL of 5 U/µL GoTaq DNA Polymerase (Promega), 0.3 µL of 40 mM dNTP mix (Promega), 0.3 µL of each primer (10 µM) (Table 1), 1 µL of DNA, and MilliQ water. PCR reactions were run in a C1000 thermocycler (BioRad) in one denaturation step at 95 °C for 3 min, 30 cycles of denaturation (95 °C, 30 s), annealing (58 °C, 30 s), and extension (72 °C, 30 s), and a final extension step (72 °C, 3 min). The PCR products were cooled to 4 °C for 5 min, then electrophoresed in a 1 % agarose gel and visualized using a UV light transilluminator (Bio-Rad). Putative haplotype W and Z bands were isolated and sequenced using Sanger sequencing (Macrogen, Korea). Further validation of the sex-differential banding patterns of Lvan_sx_PB_1 and Lvan_sx_PB_3.2 primers was done with 15 females and 16 males.

Lvan_sx_PB_1 was also used to analyze 88 larvae (44 nauplii and 44 mysis of two full-sib families) isolated under a stereoscope. For nauplii DNA extraction, individuals were macerated with a plastic pestle in 20 µL of Milli-Q water, and then heated in a microwave oven at maximum potency for 2 min in 40 s intervals (Firmansyah *et al.*, 2023). For mysis, the abdomen was excised and used for DNA isolation with a salt-extraction protocol (Aljanabi and Martinez, 1997). DNA was preserved at -20 °C without dilution as concentrations were relatively low (< 10 ng/µL in nauplii, and 20–90 ng/µL in mysis). PCR reactions were done as described above. The observed sex proportions were compared to the 1:1 expected proportion by a Chi-square analysis.

Annotation of chromosome 12 and identification of candidate sex-associated genes

From the previous work by Perez-Enriquez *et al.* (2024), we obtained a comprehensive catalog of proteins encoded by chromosome 12. Subsequently, a systematic search was conducted to identify candidate sex-linked genes. Once identified, these genes were visualized alongside the insertion-deletion (indel) described in above, using the R package chromoMap v.4.1.3 (Anand and Rodriguez-Lopez, 2022).

RESULTS

A total of 44 and 55 reads for haplotype W and haplotype Z, respectively, were aligned to CHR_12 at the SNPs associated region of Perez-Enriquez *et al.* (2020) and/or upstream and downstream (Supplementary Table S1), spanning approximately 42K bases of CHR_12 (positions 27,716,888–27,759,077). Five indels were detected in the *in silico* analysis, in which the insertion corresponded to haplotype W (Supplementary Fig.

Table 1. Primers sequences for sex differentiation in shrimp *Penaeus vannamei*, and expected band sizes depending on the haplotype. All primers were amplified at an annealing temperature of 58 °C.**Tabla 1.** Secuencias de iniciadores para la diferenciación de sexo en el camarón *Penaeus vannamei* y tamaños esperados de bandas de acuerdo con el haplotipo. Todos los iniciadores amplificaron a una temperatura de acoplamiento de 58 °C.

Name	Sequence	Expected size of PCR product	
		Haplotype W	Haplotype Z
Lvan_sx_PB_1F	5'-AGGAGCCTTTGTGCATTTAGA	487 bp	459 bp
Lvan_sx_PB_1R	5'-CATGCGCACTAGAAGTTTGA		
Lvan_sx_PB_2F	5'-GATTTGGCTCGCTGTGGAA	367 bp	314 bp
Lvan_sx_PB_2R	5'-AACCGAAAGCCATCTCCAAG		
Lvan_sx_PB_3.1F	5'-CTTCGGCGCGTAATAAGC	700 bp	167 bp
Lvan_sx_PB_3.1R	5'-ATAGAGTGAAGAGGCACGG		
Lvan_sx_PB_3.2F	5'-GATGAGGTCCTTGCCATCGC	841 bp	282 bp
Lvan_sx_PB_3.2R	5'-ACCGAAAACGCAACCTGATT		
Lvan_sx_PB_4F	5'-GATCTGGTGTGGAGCCTCT	166 bp	149 bp
Lvan_sx_PB_4R	5'-CACCTACGCTTCACACAACC		

S1).

Primers were designed for four of those indels (Table 1; Figs. 1a-d). The only indel for which no primers could be designed was an insertion in the haplotype Z that was flanked by repetitive regions (Fig. 1e).

No differential amplification between sexes was observed with primers Lvan_sx_PB_2 and Lvan_sx_PB_4 (Fig. 2). In contrast, differential amplification was evident for primers Lvan_sx_PB_1 and Lvan_sx_PB_3.1 (Fig. 2) with two bands for females (WZ) and one for males (ZZ).

Results with primers Lvan_sx_PB_1 and Lvan_sx_PB_3.1 were further validated with an additional set of samples (Fig. 3). Nevertheless, the banding pattern of Lvan_sx_PB_3.1 showed some non-specific bands (Fig. 3b), due to the possible presence of other priming sites. To improve the amplification of this region, a second set of primers (Lvan_sx_PB_3.2, Fig. 1c) resulted in a cleaner two-banding pattern for females and one band for males (Fig. 4). The Sanger sequencing of haplotypes W and Z of both Lvan_sx_PB_1 and Lvan_sx_PB_3.2 confirmed the sequences presented in Figs. 1a and 1c, respectively.

Nauplius and mysis stages Lvan_sx_PB_1 amplifications were successful, confirming that sex-identification is also possible in shrimp larvae, even with their low DNA concentration (Supplementary Figs. S2 and S3). The observed proportions of females and males at each larval stage per family did not depart from the expected values (Table 2).

A total of 239 genes were identified on the candidate sex chromosome 12 (Supplementary Table S2). Among these, 11 genes were found to be potentially involved in sex determination or sexual differentiation (Table 3). The chromosomal positions of these candidate genes on CHR_12, relative to the sex-associated SNP marker and indels, are shown in Figure 5.

DISCUSSION

We have shown the presence of indels associated with sex in *P. vannamei*. These indels are located in chromosome 12 (CHR_12) of the GCA_037577135.2 genome assembly (Perez-

Enriquez *et al.*, 2024). We have also shown that it is possible to carry out end-point PCR reactions to identify the sex of an individual, even at larval stages. This characteristic is particularly useful for studying early development sex ratios in both farmed and wild populations, for setting up adequate breeding designs (selective breeding and low inbreeding schemes) or for editing the haplotype Z to express the haplotype W, resulting in all-female individuals. The role of the identified indels in sex determination or differentiation remains to be explored.

The findings suggest that CHR_12 may function as a sex-associated chromosome in *P. vannamei*. Preliminary analyses of the genes located on this chromosome provide insights into potential candidates implicated in spermatogenesis. Notably, the gene *fd96cb* has been identified as a key factor in the proper development of male genitalia in *Drosophila melanogaster* (Vincent *et al.*, 2019). Another significant gene is *dzip*, which appears to play conserved roles in spermatogenesis across species, including rats, humans, and fruit flies. This gene is expressed in spermatogonia and contributes to ciliary membrane formation, including the development of sperm flagella (Curry *et al.*, 2006; Lv *et al.*, 2020; Wu *et al.*, 2020; Fu *et al.*, 2015; Lapart *et al.*, 2019). Although the spermatozoa of *P. vannamei* lack a flagellum structure (Alfaro-Montoya *et al.*, 2016), their function justifies further investigation to elucidate their role in the spermatogenesis processes.

The *crem* gene plays a key role in mouse spermiogenesis by regulating over 4,700 testicular genes, including 101 associated with spermatogenesis. Mutations in *crem* result in the absence of late spermatids and increased apoptosis in mice (Nantel *et al.*, 1996; Sassone-Corsi, 1998; Peri and Serio, 2000; Beißbarth *et al.*, 2003; Kosir *et al.*, 2012; Sánchez-Jasso *et al.*, 2023). The *hspa2* gene, also annotated, is essential for spermatogenesis in humans and mice, with its deficiency causing spermatogenic arrest at the G2/M phase of meiosis I and severe damage to the seminiferous epithelium (Son *et al.*, 1999; Son *et al.*, 2000; Scieglinska and Krawczyk, 2015).

The *zfy* gene, a zinc finger transcription factor located

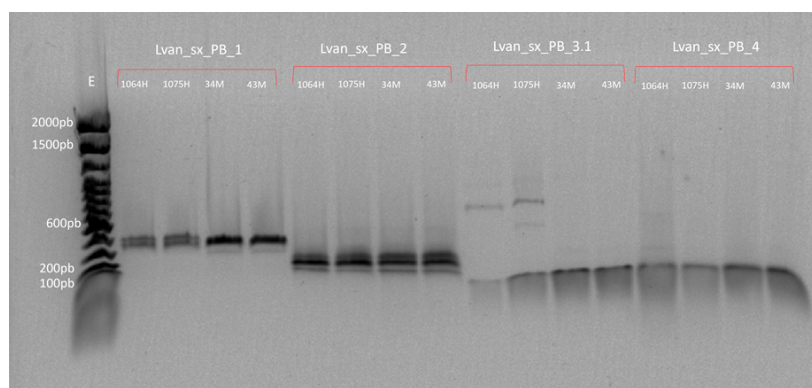
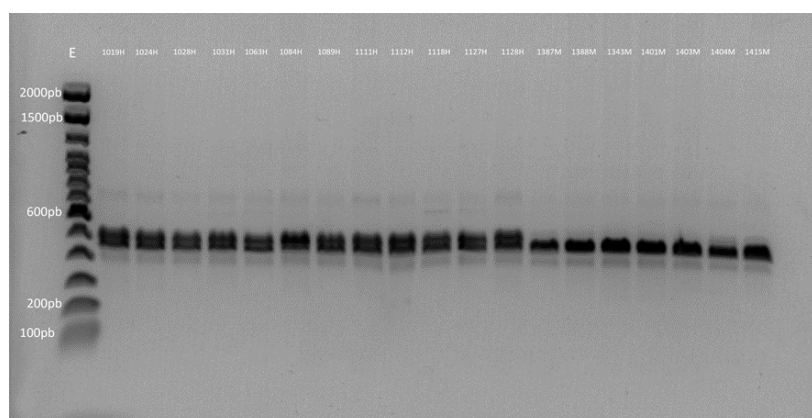


Fig. 2. Females (H) and males (M) amplifications with primers Lvan_sx_PB_1, Lvan_sx_PB_2, Lvan_sx_PB_3.1, and Lvan_sx_PB_4. E: 100 bp DNA ladder.

Fig. 2. Amplificaciones de hembras (H) y machos (M) con los iniciadores Lvan_sx_PB_1, Lvan_sx_PB_2, Lvan_sx_PB_3.1 y Lvan_sx_PB_4. E: escalera de peso molecular de 100 pb.

a)



b)

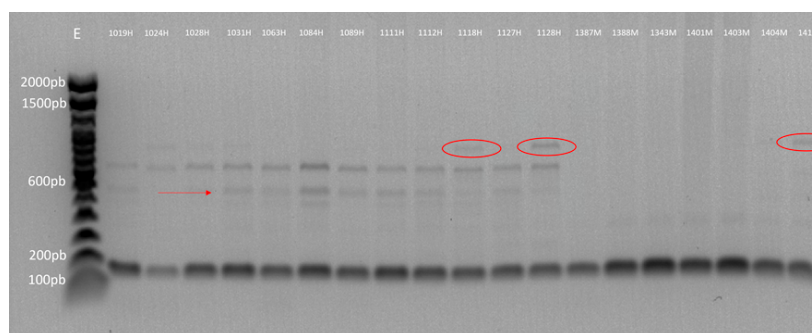


Fig. 3. Females (H) and males (M) amplifications with primers Lvan_sx_PB_1 (a), and Lvan_sx_PB_3.1 (b). E: 100 bp DNA ladder. Arrow and red circles indicate non-specific bands.

Fig. 3. Amplificaciones de hembras (H) y machos (M) con los iniciadores Lvan_sx_PB_1 (a) y Lvan_sx_PB_3.1 (b). E: escalera de peso molecular de 100 pb. La flecha y elipses rojas indican bandas no específicas.

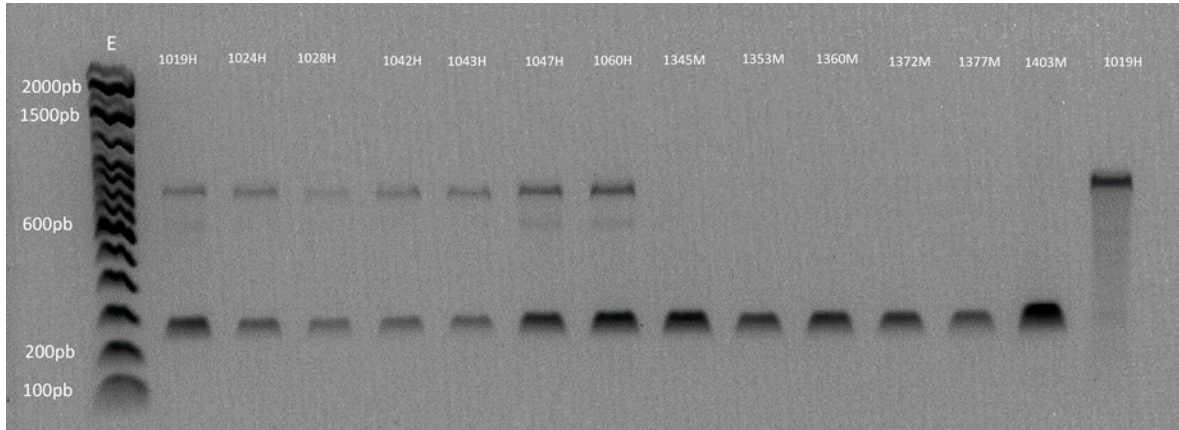


Fig. 4. Females (H) and males (M) amplifications with primer Lvan_sx_PB_3.2. E: 100 bp DNA ladder. The last sample to the right shows a purified fragment of the putative haplotype W.

Fig. 4. Amplificaciones de hembras (H) y machos (M) con el iniciador Lvan_sx_PB_3.2. E: escalera de peso molecular de 100 pb. La última muestra de la derecha es el fragmento purificado del haplotipo W putativo.

Table 2. Sex proportion of nauplii (larval stage N5) and mysis (larval stage M3) per family determined by the amplification of primer Lvan_sx_PB_1. NA: No amplification. χ^2 : Chi-square (none of the values was higher than $\chi^2_{(0.05,1)} = 3.84$).

Tabla 2. Proporción de nauplios (estadio larval N5) y mysis (estadio larval M3) por familia determinado por la amplificación del iniciador Lvan_sx-PB_1. NA: No amplificó. χ^2 : Chi-cuadrada (ninguno de los valores fue mayor que $\chi^2_{(0.05,1)} = 3.84$).

Family	Larval stage	Males	Females	NA	χ^2
F1 ^a	N5	12	9	1	0.43
	N5	11	11	0	0
F3	M3	12	8	2	0.8
	N5	13	9	0	0.73
F5	M3	11	9	2	0.2

^a Mysis from F1 amplified in few individuals.

Table 3. Potentially sex-associated genes found in chromosome 12 of the genome assembly of Perez-Enriquez *et al.* (2024).

Tabla 3. Genes potencialmente asociados al sexo localizados en el cromosoma 12 del ensamblaje genómico de Perez-Enriquez *et al.* (2024)

Gene identifier	Uniprot entry name	Protein name	start	end
g6043.t1	S39A9_HUMAN	Zinc transporter ZIP9	497042	506834
g6051.t1	FD5_DROME	Fork head domain-containing protein FD5	3638107	3639783
g6120.t1	DZIP1_DANRE	Cilium assembly protein DZIP1	12834098	12835146
g6125.t1	FEM1C_BOVIN	Protein fem-1 homolog C	13243880	13441732
g6176.t1	CREM_CANLF	cAMP-responsive element modulator	19844271	19845141
g6234.t1	HSP72_HUMAN	Heat shock-related 70 kDa protein 2	24827835	24828871
g6285.t1	PROF_DROME	Profilin	28515906	28531810
g6299.t1	ZFY_PIG	Zinc finger Y-chromosomal protein	30463341	30463613
g6380.t1	DHC24_HUMAN	Delta(24)-sterol reductase	37090104	37093780
g6460.t1	SO_DROME	Protein <i>sine oculis</i>	43813704	43814381
g6482.t1	BMP7_HUMAN	Bone morphogenetic protein 7	45869361	45869678

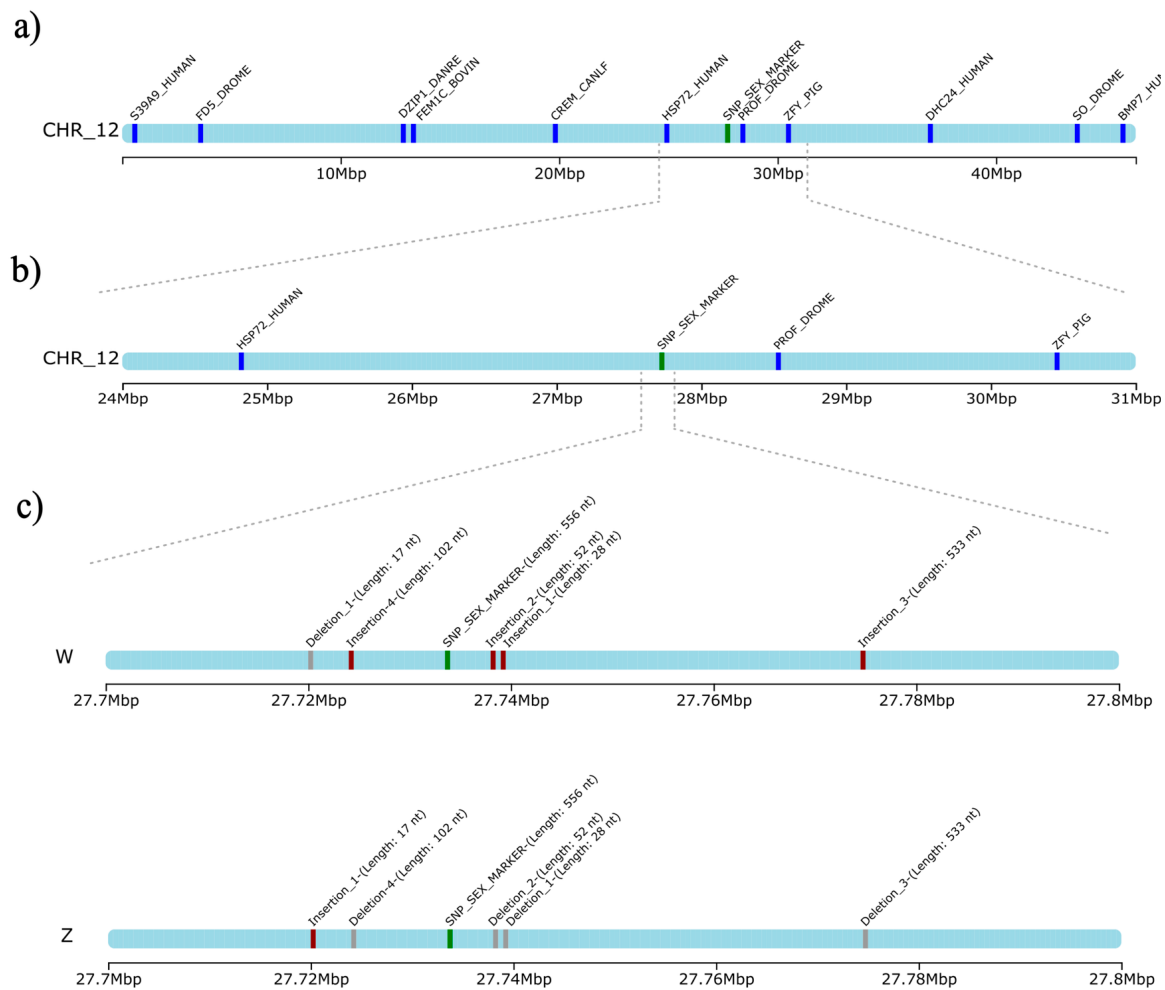


Fig. 5. Protein annotation of chromosome 12. a) Comprehensive annotation of relevant sex-associated proteins (Table 3). b) Detailed view of the location of the SNP sex marker (Perez-Enriquez *et al.*, 2020). c) Location of insertions/deletions (Fig. 1) in the W and Z haplotypes. Blue bars denote protein-coding genes names; red and gray bars indicate insertions and deletions, respectively.

Fig. 5. Anotación de proteínas del cromosoma 12. a) Anotación completa de proteínas relevantes asociadas al sexo (Tabla 3); b) Vista detallada del marcador de sexo tipo SNP (Perez-Enriquez *et al.*, 2020); c) Localización de las inserciones/delecciones (Fig. 1) en los haplotipos W y Z; Las barras azules denotan los nombres de los genes que codifican proteínas; las barras rojas y grises indican inserciones y deletiones, respectivamente.

on the Y chromosome in humans and mice, was also identified. In mice, *zfy* comprises two isoforms, *zfy1* and *zfy2*, and their knockout leads to male infertility, sperm head and tail malformations, and acrosomal abnormalities (Yamauchi *et al.*, 2022). The *zfy* gene is implicated in meiotic sex chromosome inactivation and the elimination of cells with unpaired chromosomes during the first meiotic division (Vernet *et al.*, 2016; Holmlund *et al.*, 2023). Although paralogs of *zfy* were identified on other *P. vannamei* chromosomes (data not shown), its presence on CHR_12 merits further investigation to clarify its role in sex differentiation.

The *dhcr24* gene, identified in *Macrobrachium nipponense*, encodes an enzyme critical for cholesterol biosynthesis

and reproductive regulation. It is expressed across multiple tissues, with the highest levels in the testis, specifically in spermatogonia and spermatocytes. RNA interference (RNAi) studies revealed that *dhcr24* silencing does not alter sex ratios but increases 17 α -methyltestosterone levels, suggesting its role in converting this synthetic androgen to cholesterol. This gene is proposed to contribute to sex differentiation and gonadal development by regulating steroid synthesis, thereby influencing the male reproductive endocrine axis, including the X-organ-sinus gland, androgenic gland, and testes (Cai *et al.*, 2024).

Three more genes implicated in spermatogenesis were annotated: *sine oculis* (*so*) and *chickadee* (*chic/profilin*) in *D.*

melanogaster, and *bone morphogenetic protein 7 (bmp7)* in *Eriocheir sinensis*. The first two, *so* and *chic*, play critical roles in the development of spermatocytes in *Drosophila*. These genes are expressed in somatic cyst cells, which encapsulate germ cells and deliver essential regulatory signals during spermatogenesis. Knockout of both genes has been demonstrated to induce defective spermatogenesis (Fabrizio *et al.*, 2003; Fairchild *et al.*, 2015). In contrast, *bmp7* in *E. sinensis*, a crustacean species, exhibits predominant expression within the testis, with *in situ* hybridization revealing its localization in spermatogonia and mature sperm, although at reduced levels in spermatocytes and spermatids, suggesting a significant stage-specific role in spermatogenesis (Xu *et al.*, 2018).

Interestingly, we also found the presence of the *fem-1c* gene, which is essential for male sexual development in *Caenorhabditis elegans*, acting in both somatic and germline sex determination (Hodgkin, 1986). Given its significance, this gene has been extensively investigated in crustaceans. In *E. sinensis*, three *Esfem-1* isoforms (*Esfem-1a*, *Esfem-1b*, and *Esfem-1c*) have been characterized. These isoforms are expressed during early embryonic development, suggesting potential maternal inheritance. Although their transcripts are detected across multiple tissues, expression levels are markedly elevated in the hepatopancreas, muscle, ovary, and testis (Song *et al.*, 2015). Notably, the *Esfem-1c* isoform plays a critical role in both ovarian and testicular development, as its depletion leads to altered female gonopores and male genitalia in juvenile crabs (Zhu *et al.*, 2023). In *M. nipponense*, the *Mnfem-1* transcript is specifically expressed in the ovary and during early embryonic stages, supporting the hypothesis of maternal transcript transfer and suggesting a role in sex determination and differentiation mechanisms (Ma *et al.*, 2016). Similarly, in *P. vannamei*, *PvFem-1c* is proposed to be maternally transferred, as evidenced by its expression during early embryonic development. Although *PvFem-1c* is expressed in both gonads, a natural antisense transcript in the oocyte nucleus has been proposed as a mechanism for regulating its expression through silencing (Galindo-Torres *et al.*, 2019). In *C. elegans*, the *fem-1* transcript is maternally transferred to the progeny via oocytes (Doniach and Hodgkin, 1984) and is implicated in the epigenetic regulation of *fem-1* during early zygotic expression. This process involves providing seed material for silencing RNAs, enabling the embryonic genome to express and translate the *fem-1* gene (Johnson and Spence, 2011). In contrast, the role of *fem-1* in crustaceans remains less clear, with functions in both male and female development still under investigation. For instance, recent studies in *P. vannamei* have identified two additional transcripts, *PvFem-1a* and *PvFem-1b*, alongside *PvFem-1c*, which exhibit sex-biased expression from the limb bud embryo (LBE) to postlarval stages. These three transcripts are predominantly expressed in males from LBE to Zoea III, whereas in females, expression is higher from Mysis I to Postlarva I. Furthermore, elevated expression in the ovary suggests a potential role in ovarian development (Yu *et al.*, 2023). Although the precise role of *Pvfem-1c* in sex determination and differentiation in

P. vannamei remains unclear, the mapping of the *C. elegans fem-1* ortholog in *P. monodon* to a genomic region identified as a sex-determining locus through quantitative trait locus (QTL) analysis (Robinson *et al.*, 2014), is consistent with observations in *P. vannamei* (Perez-Enriquez *et al.*, 2020). This feature suggests that this gene may play a significant role in sex determination and/or differentiation.

CONCLUSIONS

We have reported sex-associated indels in *P. vannamei*, not previously described, and used them to develop a simple protocol for DNA-based sex identification. The mapping of these indels [and the sex-associated SNPs (Perez-Enriquez *et al.*, 2020)], to chromosome 12 [GCA_037577135.2 genome assembly (Perez-Enriquez *et al.*, 2024)], along with several sex-related genes, suggests that this chromosome is relevant for sex determination and/or differentiation. Nevertheless, since other sex markers (e.g., Yu *et al.*, 2017; Jones *et al.*, 2020; Wang *et al.*, 2020) align with different chromosomes, further work is needed to confirm chromosome 12's role in sex differentiation and to uncover the presence of potential sex-determination mechanisms.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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